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Mutagenic By-Products from Chlorination of Humic Acid

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Chlorination of humic and fulvic acid results in the formation of direct-acting mutagenicity, detectable in the Salmonella/microsome assay (Ames test). This mutagenicity is being characterized as part of an overall effort aimed at evaluating potential health risks associated with the presence of mutagenic chemicals in drinking water. A number of chlorinated organic compounds, including several known mutagens, have been identified and quantified in diethyl ether extracts of chlorinated humic acid solutions. However, the total mutagenicity of these compounds accounts for only about 7% of the original mutagenicity. Synergistic or antagonistic interactions among the identified components have been ruled out as possible explanations for the failure to account for a higher percentage of the activity. Recent progress has been made to separate the activity into neutral and strong acid fractions. Further isolation of the strong acids by high-pressure liquid chromatography (HPLC) has resulted in the purification of the mutagenicity into a major peak of activity with a specific mutagenicity of about 20,000 TA100 revertants per milligram. Several trichlorohydroxyfuranone isomers have been tentatively identified in this fraction. The contribution of these types of compounds to the mutagenicity of chlorinated humic acid is under investigation.

Introduction

Until about 10 years ago, concern about toxic chemicals in drinking water centered on the problem of contamination of our water supplies from various industrial and municipal discharges. This concern certainly remains alive today, but the emphasis on the problem of organic contamination of surface waters has changed considerably as a result of the discovery of trihalomethanes in drinking water in 1974 (*1, 2*). Since then, we have come to realize that many organic contaminants, particularly halogenated compounds, originate during water disinfection as a result of reactions of chlorine with naturally occurring humic substances.

Currently, more than 1100 organic compounds have been identified in drinking water, most at or below the μg/L level (*3*). For most of the compounds, information on the genotoxic or carcinogenic properties is lacking (*4*). A substantial portion of the compounds that have been tested for genotoxicity have been found to be active (*4, 5*). In addition, a number of studies have demonstrated the widespread occurrence of mutagenic activity in uncharacterized, organic concentrates of finished drinking water (*6, 7*). These concentrates consist primarily of nonvolatile organics, which are relatively refractory to identification. In view of these findings, it is apparent that the contamination of drinking water with mutagenic chemicals is a very complex problem.

The critical question becomes whether the presence of mutagenic chemicals in drinking water represents a significant threat to human health. An understanding of the processes that contribute to the occurrence of the mutagens should help in the eventual identification of compounds that are important contributors to mutagenicity. In a number of studies, water chlorination has been shown to be directly responsible for the production of mutagenic compounds in water (*8–10*). Previous work in our laboratory strongly suggests that this mutagenicity is largely caused by reactions of chlorine with humic substances (*11–14*). The present work summarizes our progress to date characterizing the mutagenicity of humic acid chlorination by-products.

Materials and Methods

Chemicals

Solutions of humic acid (Fluka Chemical Co.) were prepared at a concentration of 1 g total organic carbon (TOC) per liter distilled water and chlorinated at an initial pH of 7.0 using a 1:1 molar ratio of Cl:C. Following the addition of chlorine, the solutions were allowed to stand for 90 hr at room temperature to ensure complete

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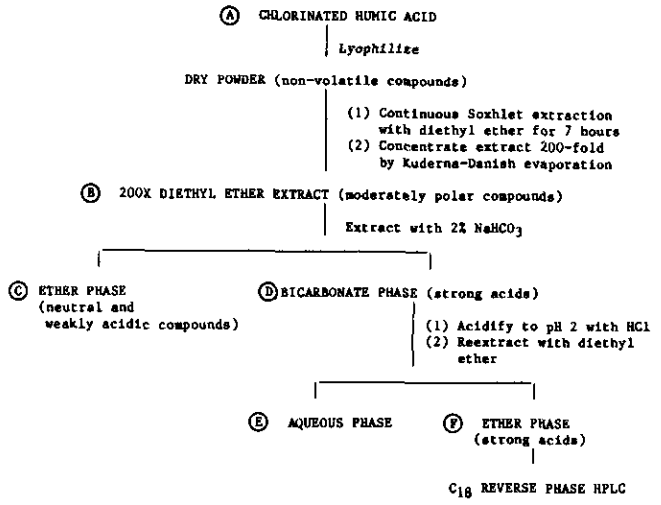


FIGURE 1. Scheme for fractionation of mutagenic components of *chlorinated humic acid.*

reaction, and then were stored at 4°C. The detailed procedures, as well as the rationale for choosing these reaction conditions, are discussed elsewhere (*11,12*). *N*-Methyl-*N*-(*tert*-butyldimethylsilyl) trifluoroacetamide (MTBSTFA) was obtained from Regis Chemical Co. Mucochloric acid was purchased at 99% purity from Aldrich Chemical Co. All other chemicals were either synthesized in our laboratory or obtained from commercial sources, as indicated elsewhere (*13*).

Extraction and Fractionation of Mutagenic Components

Extraction and concentration of the chlorinated humic acid solutions for compound identification and quantification studies were accomplished by a liquid–liquid extraction procedure using diethyl ether as solvent (*13*). A second extraction procedure, modified from the method of Holmbom (*15*), was applied to lyophilized chlorinated humic acid. This method was used to obtain a fraction containing the strong acid components. The fractionation procedure is depicted in Figure 1. The final ether extract, representing the strong acid fraction, was subjected to reverse-phase, high-pressure liquid chromatography (HPLC) for further isolation of mutagenic components. Two different HPLC conditions were tried. The first used a C_{18} analytical column (Varian Microprobe MCH-10, 10 μm particle size, 30 cm × 4 mm) and a 20–100% acetone-in-water, linear gradient for 30 min. Fractions were collected every 2.5 min. The second method used a C_{18} semipreparative column (Dupont Zorbax ODS, 5 μm particle size, 25 cm × 9.4 mm) and a 30–100% methanol-in-water, linear gradient for 30 min, followed by 100% for 10 min, and a return to 30% in 20 min. Fractions were collected every 2 min. In both cases, the flow rate was 1 mL/min. In the case of the semipreparative HPLC run, the fractions were monitored for absorbance at 260 nm and 400 nm, total organic halogen (TOX) content, residue weight, and mutagenicity in the Ames test. Absorbance readings were made using a Beckman Model 35 UV-VIS spectrophotometer. TOX was measured by the granular activated carbon (GAC) adsorption-microcoulometric method by using a Dohrmann Total Organic Halogen Analyzer. Residue weights were determined in duplicate by adding 200 μL sample aliquots to preweighed aluminum weigh boats in an evacuated dessicator and weighing 24 hr later using an analytical balance.

Assay for Mutagenic Activity

The Salmonella/microsome mutagenicity assay developed by Ames et al. (*16*) and later modified by Maron and Ames (*17*) was used to test for mutagenic activity. *S. typhimurium* strains TA 98 or TA 100 were used in these studies. HPLC fractions were assayed in the absence of metabolic activation at 50- and 100-μL doses using duplicate plates per dose. Other details of our test procedure have been previously described (*13*). In some experiments, the assay pH was altered by adjusting the pH of the base agar to either 6 or 8 by the method used by Popkin and Prival (*18*).

Results and Discussion

A wide variety of halogenated products resulting from humic acid chlorination have been previously identified in our laboratory using liquid–liquid extraction, closed-loop stripping, or steam distillation techniques in conjunction with gas chromatography-mass spectrometry (GC-MS) analysis (*12–14*). The compounds identified include halogenated aldehydes, acids, ketones, nitriles, alkenes, and aromatic compounds. Fourteen of the forty-two identified compounds are known to be mutagens, whereas many others apparently have not been tested for mutagenicity (*14*). The mutagenic activities in strain TA 100 of the compounds for which quantifications have been made are listed in Table 1. Assuming additivity of response, these data indicate that the identified and quantified mutagenic constituents account for only about 7% of the total TA 100 mutagenicity. Other experiments have shown that synergism among the components does not appear to be a major factor in the failure to account for a higher percentage of the activity (*12*).

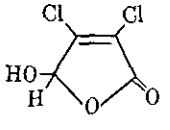
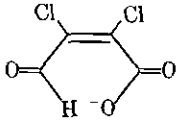
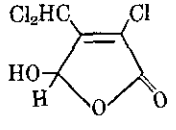
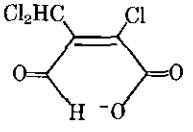
Recent work by Holmbom et al. (*15*) suggests that a major part of the mutagenicity of pulp mill chlorination stage effluents is caused by a compound tentatively identified as 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5H)furanone, which they term MX. This finding was of interest to us because humic material is thought to be derived from lignin (*19*) and because of the similarities of the by-products that are formed from reactions of chlorine with these two substrates. To our knowledge, MX has not been synthesized, but the results of Holmbom et al. (*15*), based on testing highly purified fractions containing the compound, indicate that it is a very potent mutagen (*15*). MX is structurally similar to

Table 1. Contribution of identified mutagenic compounds to the total mutagenicity of a chlorinated humic acid sample.[a]

Compound	Concentration, mg/L	Specific activity for TA 100, net revertants/mg	Theoretical contribution to mutagenicity, net revertants/mL[b]	% Total
Dichloroacetonitrile	5.3	645	3.42	0.13
1,1-Dichloropropanone	1.0	38	0.04	<0.01
1,3-Dichloropropanone	0.15	113,900	17.08	0.67
1,1,1-Trichloropropanone	9.8	753	7.38	0.29
1,1,3-Trichloropropanone	0.05	24,633	1.23	0.05
1,1,3,3-Tetrachloropropanone	2.6	7,790	20.31	0.80
Pentachloropropanone	7.2	3,710	26.71	1.05
3,3-Dichloropropenal	.06	5,830	0.35	0.01
2,3,3,-Trichloropropenal	.035	2,560,000	84.60	3.53
Sum of identified compounds			166.12	6.55
Total sample activity[b]			2537 ± 196	(100.00)

[a] Adapted from Meier et al. (*13*).
[b] Mean slope value ± standard deviation of the mean.

Table 2. Mutagenic activities of mucochloric acid and MX in the Ames test.

Compound	Structure pH < 7	Structure pH > 7	TA 100 (−S9), net revertants/nmole[a]
Mucochloric acid			1.3[b]
3-Chloro-4-(dichloromethyl)-5-hydroxy-2(5H) furanone (MX)			2,800–10,000[c]

[a] Assays were conducted at pH 7.
[b] Based on slope of dose-response curve (−S9) in Figure 2.
[c] According to Holmbom et al. (*15*).

mucochloric acid (Table 2), which has also been reported to be mutagenic by Rapson et al. (*20*). Since only qualitative mutagenicity data were presented by Rapson et al. (*20*), we decided to determine the mutagenic potency of mucochloric acid in the Ames test with the TA 100 strain. The results (Fig. 2) confirm the direct-acting mutagenicity of this compound. The addition of a rat liver metabolic activation system (+S9) to the assay reduced the level of activity at low doses, but extended the range at which activity was detectable, apparently because of a reduction in the cytotoxic effects at high doses. The mutagenic potency was estimated to be about 2,000- to 10,000-fold lower than that calculated by Holmbom for MX (Table 2). If confirmed by testing the pure MX compound, this result would indicate that the dichloromethyl substituent is very important for the mutagenicity of MX. The allylic chlorine, being an excellent leaving group, should facilitate nucleophilic substitutions at the dichloromethyl group with DNA.

Halogenated hydroxyfuranones, such as mucochloric acid and MX, exhibit a pH-dependent tautomerism as shown in Table 2. At neutral pH and above, ring opening and ionization occur. This tautomerism explains why these compounds behave as strong acids. We attempted to determine whether the mutagenicity in chlorinated humic acid is associated with strongly acidic compounds using an extraction scheme (Fig. 1) similar to the one developed by Holmbom, using diethyl ether rather than ethyl acetate as a solvent. The mutagenic activity and organic halogen content were determined for each of the fractions (A through F). The results (Table 3) indicate that about 43% of both the TA 100 mutagenicity and organic halogen content, originally extractable in ether (fraction B), was recovered in the strong acid fraction. Of the activity, 19% remained in the ether phase following bicarbonate extraction (fraction C), presumably associated with neutral or weakly acidic compounds. Only about 3% of the TOX was recovered in this fraction. It is notable that very little mutagenicity appeared initially to be extractable into the bicarbonate phase (D_1), but following acidification of this fraction (D_2) and reextraction into ether (F), the mutagenicity

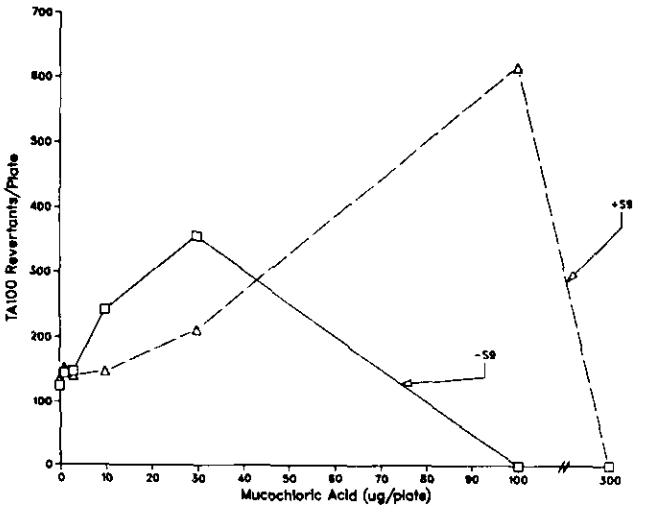


FIGURE 2. Mutagenicity of mucochloric acid for strain TA 100 in the Ames test. Assays were conducted at pH 7.

increased dramatically. If the mutagenicity in the strong acid fraction is associated with chlorinated hydroxyfuranones, it would appear that these compounds are most active in their closed, lactone form. This possibility was investigated with mucochloric acid, which we have recently identified (confirmed) by methylation of the strong acid fraction. The effect of adjusting the assay pH on the mutagenicity of mucochloric acid can be seen in Table 4. At pH 6, a mutagenic response of greater than twice background was observed at a dose of 30 nmole/plate, whereas at higher doses, toxicity was evident, as judged by the presence of pinpoint colomes and substantial clearing of the background lawn. The level of mutagenicity in terms of revertants per nmole, induced by mucochloric acid at pH 6, appeared to be about five times that observed in the assay at pH 7 and about 10 times that observed in the assay at pH 8. No toxicity was apparent at pH 7 or 8, even at doses of 120 nmole/plate. Thus, the apparent pH dependence of the mutagenicity is consistent with the idea that mucochloric acid is more active in its lactone form. The reason for this pH dependency is unclear but may be related to decreased uptake of the ionized form into the cell.

To further isolate the mutagenic activity of chlorinated humic acid, the strong acid fraction was subjected to C_{18} reverse-phase HPLC. Because the organic material appeared to be largely soluble in acetone, we initially tried an acetone-in-water gradient for the mobile phase, using an analytical column. Two peaks of mutagenicity were apparent, a major peak in fraction 6 and a minor peak in fraction 9 (Figure 3). Several additional HPLC runs were made, and the fractions containing the major peak of activity were pooled. This pooled material was then derivatized either by methylation (*21*) or silylation using MTBSTFA before GC/MS analysis. Interpretation of the mass spectra of the major gas chromatographic peaks indicated the presence of several compounds with mass fragmentation patterns consistent with chlorinated hydroxyfuranone structures. The structural possibilities based on manual interpretation of the mass spectra are shown in Figure 4. Two of the compounds appear to be unsaturated at the 3,4 position, and several more appear to be saturated at this position. At present, we cannot determine whether the methyl group is at the 3 or 4 carbon position or whether the chlorine atoms are all on the same carbon.

To obtain larger quantities of the isolated mutagenic material, a semi-preparative HPLC column was used. In this case, the acid fraction was transferred into 30% methanol, and a mobile phase of methanol in water was used. This procedure was used because we found that 85% of the mutagenicity was recovered when the solvent was transferred from ether to 30% methanol, even though a large precipitate was evident. The use of methanol as a mobile phase had the additional advantage over acetone in that the ultraviolet absorbance of the fractions could be read with minimal interference from the solvent. The results of the semipreparative HPLC sep-

Table 3. Distribution of mutagenicity and organic halogen content of chlorinated humic acid following sequential ether-bicarbonate-ether extractions.

Fraction		TA 100 mutagenicity		Total organic halogen	
		Net revertants/mL[a,b]	% Fraction B	mg/L[b,c]	% Fraction B
A	Original sample	3834 ± 166	—	451	—
B	Diethyl ether extract of lyophilized original	3009 ± 54	(100)	293	(100)
C	Ether phase after bicarbonate extraction of B	580 ± 63	19	9.7	3.3
D_1	Bicarbonate phase after bicarbonate extraction of B	(232 ± 55)[d]	8	146	49.8
D_2	Acidified fraction D_1	815 ± 62[e]	27	146	49.8
E	Bicarbonate phase after diethyl ether extraction of D_2	NS	—	25	8.6
F	Diethyl ether extract of D_2	1286 ± 80	43	124	42.2

[a] Initial slopes ± standard error of the slope of the dose-response curve.
[b] Values are relative to the volume of the original sample.
[c] Mean value from duplicate determinations.
[d] Response less than twofold above background at highest dose, but clear evidence of an increasing dose-response.
[e] Not significant (i.e., no evidence of a dose-response and response at any dose less than twofold above background).

Table 4. Effect of assay pH on the mutagenicity of mucochloric acid.

Assay pH	Dose level, nmole/plate	TA 100 revertants per plate[a]	TA 100 net revertants per nmole[b]	% of activity at pH 6
6	0	146 ± 15	5.53	(100)
	30	312 ± 12		
	60	toxic		
	90	toxic		
	120	toxic		
7	0	156 ± 13	1.17	21
	30	206 ± 17	± 0.12	
	60	247 ± 1		
	90	277 ± 29		
	120	294 ± 22		
8	0	108 ± 15	0.54	10
	30	129 ± 10	± 0.08	
	60	137 ± 16		
	90	156 ± 7		
	120	175 ± 20		

[a] Mean value ± standard deviation from triplicate plates.
[b] Calculated based on response at 30 nmole per plate for the pH 6 assay and from slope of dose-response curves at pH 7 and pH 8.

aration are shown in Figure 5. Similar to the previous analytical separation, two distinct peaks of mutagenicity were evident, a major peak in fraction 16 (61% of the activity) and a minor peak in fractions 19–20 (29% of the activity). The mutagenicity was well resolved from most of the residue weight and the organic halogen, which co-eluted in fractions 10–12. Trichloroacetic acid, a major by-product of humic acid chlorination, also eluted in fractions 10–12, as determined by injection of the pure compound alone and monitoring for TOX. In contrast to the well-defined separation of mutagenicity

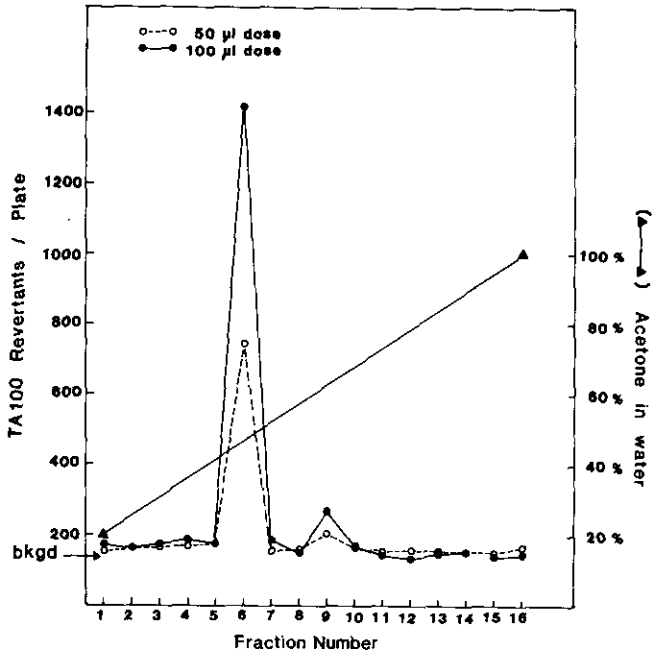


FIGURE 3. Analytical HPLC separation of mutagenic activity in strong acid fraction of chlorinated humic acid.

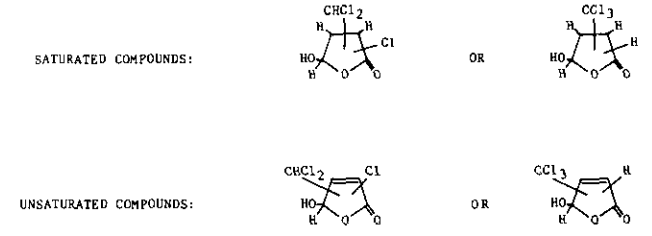


FIGURE 4. Chlorinated hydroxyfuranones tentatively identified in the major mutagenic fraction from the analytical HPLC separation of the strong acid fraction of chlorinated humic acid.

from the bulk of the weight and the organic halogen associated with the acid fraction, the yellow-colored (A_{400}) and UV-absorbing (A_{260}) material eluted in several broad, poorly defined peaks, covering fractions 11–20.

The specific mutagenicities of the active fractions were also calculated. The specific mutagenicities (TA 100 net revertants per mg) were: 19,900 for fraction 16; 19,500 for fraction 19; and 27,800 for fraction 20. The values for the original ether extract of lyophilized chlo-

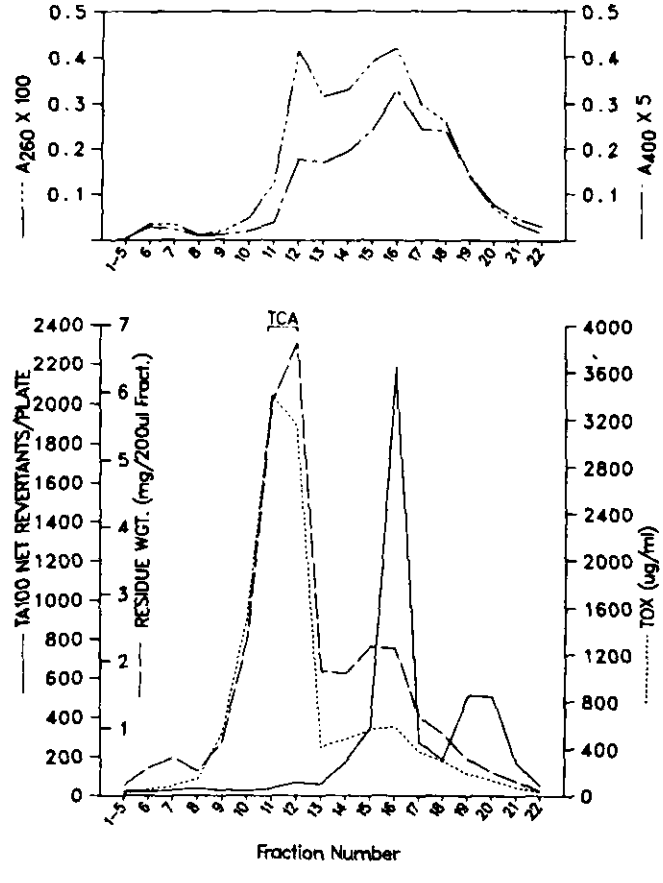


FIGURE 5. Semipreparative HPLC analysis of the strong acid fraction of chlorinated humic acid.

Table 5. Major compounds detected in the mutagenic peaks from semipreparative HPLC of the strong acid fraction of chlorinated humic acid.

	Compound
16	Dichloromethyl-dichloropropenoic acid[a]
	Trichlorooxobutanoic acid[a]
	1,1,3,3-Tetrachloropropanone
	Pentachloropropanone
	Trichlorocyclopentenedione
	Methyl-trichloromethyldioxanedione
	Methyl chloronaphthoate
19-20	Trichlorooxopentenoic acid (two isomers)[a]
	Tetrachlorooxopentenoic acid[a]
	Trichlorotetradecadienoic acid[a]
	1,1,3,4,5,5,5-heptachloropent-3-en-2-one

[a] Identified as the dimethyl-*tert*-butylsylyl (DMTBS) ester.

rinated humic acid (Fraction B, Table 3) and for the strong acid fraction (Fraction F, Table 3) after transfer into 30% methanol, were 2,100 and 2,950 revertants per milligram, respectively. Thus, the purity of the major portion of mutagenic material on an organic residue weight basis was increased about tenfold by the overall solvent extraction/HPLC fractionation procedures.

GC-MS analysis of the mutagenicity-active HPLC fractions resulted in the tentative identification of several chlorinated ketones, as well as both saturated and unsaturated chlorinated aliphatic keto acids (Table 5). Many of the latter compounds have also been recently identified by deLeer et al. as humic acid chlorination products (*22*). Several chlorinated hydroxyfuranones were also detected in fraction 16, but these appeared as minor peaks in the GC chromatogram. It appears that further isolation of the mutagenic material will be required before the importance of the chlorinated hydroxylfuranones or the other compounds in the mutagenic HPLC peaks can be assessed definitively.

In spite of the extensive investigation into the problem of mutagens in drinking water by researchers around the world, we still do not really know whether the presence of such mutagenicity represents a significant genetic or carcinogenic risk to the human population. Despite our present lack of knowledge regarding the magnitude of risk, and in view of the potential risks, it would seem prudent to undertake measures to reduce our exposure to the mutagens as much as possible. Reducing our exposure requires that we gain a better understanding of the identity, levels, frequency of occurrence, and origin of the compounds responsible for the mutagenicity of drinking water. Only by understanding the processes that contribute to the formation and occurrence of mutagenic chemicals can we intelligently decide how to eliminate them. Furthermore, the identification of those compounds that are important contributors to the mutagenic activity of drinking water should provide a way of ranking chemicals for further testing in the more expensive animal carcinogenesis bioassays that are currently used to develop regulatory standards for carcinogens.

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